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Article (Published Version)

Newcombe, Sonya, Bobin, Mariusz, Shrikhande, Amruta, Gallop, Chris, Pace, Yannick, Yong, Helen, Gates, Rebecca, Chaudhuri, Shuvashri, Roe, Mark, Hoffmann, Eva and Viseux, Eddy M E (2013) Gold amides as anticancer drugs: synthesis and activity studies. *Organic and Biomolecular Chemistry*, 19 (11). pp. 3255-3260. ISSN 1477-0520

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Gold amides as anticancer drugs: synthesis and activity studies†

Cite this: DOI: 10.1039/c3ob27460h

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Accepted 26th March 2013

DOI: 10.1039/c3ob27460h

www.rsc.org/obc

Modular gold amide chemotherapeutics: Access to modern chemotherapeutics with robust and flexible synthetic routes that are amenable to extensive customisation is a key requirement in drug synthesis and discovery. A class of chiral gold amide complexes featuring amino acid derived ligands is reported herein. They all exhibit *in vitro* cytotoxicity against two slow growing breast cancer cell lines with limited toxicity towards normal epithelial cells.

Introduction

Metal complexes have been used for medical purposes for almost 5000 years.^{1,2} In the past 20 years, gold(i) and gold(iii) complexes have been investigated as potential anticancer drug candidates.³ Two compounds – auranofin and sodium aurothiomalate – (Fig. 1) are currently undergoing phase II and phase I clinical trials for treatment of lymphomas and non-small cell lung cancer, respectively. Aurothiomalate reportedly interacts with PKC α whereas auranofin inhibits thioredoxin reductase (TrxR) *via* a ligand exchange mechanism with the selenocysteine residue within the active site of the protein. TrxR plays a major role in the regulation of the cellular redox state and is over-expressed in some tumours.^{4,5}

The structures of gold(i) complexes L–Au–X are linear in nature and feature two ligands, L and X. The L-ligands can be phosphines, *N*-heterocyclic carbenes (NHCs) or sulfides, whereas the X-ligands range from thiolates, featured notably in the anti-rheumatoid auranofin, bis-triflic amide, chlorides, alkoxides, sulfonates and NHCs.^{5–13} Previously the structural nature of ligands L and X limited their potential for derivatization and the corresponding complexes exhibited significant cytotoxicity. We have developed two complementary approaches that combine biocompatible ligands with tunable lipophilic groups to provide a platform for anti-cancer drug

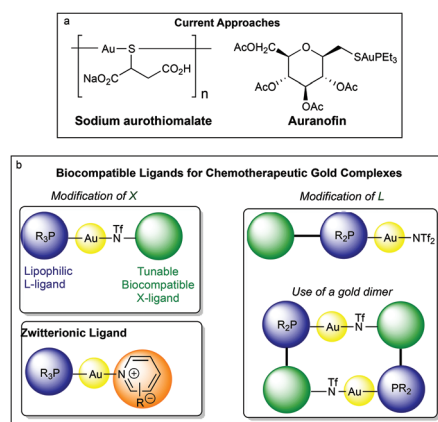


Fig. 1 Current and novel approaches to chemotherapeutic gold(i) complexes. (a) Sodium aurothiomalate and auranofin are currently in clinical trials for treatment of lymphomas and lung cancer. (b) The development of gold amide drugs allows easy access to tunable gold complexes featuring biocompatible ligands.

discovery. In the first approach, we take advantage of the varying physical properties of amino acids to derivatize biocompatible ligands bound to gold monomers or dimers (Fig. 1). In the second approach, we used a polarized gold(i) complex which features a zwitterionic ligand containing a delocalized pyridinium cation (Fig. 1). We hypothesized that a polarized complex would have enhanced efficacy at killing adenocarcinoma-derived cells, as these are generally hyperpolarized as discussed in the Results section.

We have demonstrated that these complexes bearing the common triflic amide motif, yet featuring a variety of structural backbones, can act as potent inhibitors of two breast cancer cell lines *in vitro*. This validated approach contrasts with previous use of delocalized lipophilic cations (DLCs),¹⁰ as the X-ligand provides adjustable lability and physical

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†Electronic supplementary information (ESI) available: Experimental and spectroscopic details for all compounds are included, including X-Ray diffraction spectra for all complexes. CCDC 915632–915634. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c3ob27460h

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properties ideal for cell permeation. The gold amide linkage allows the conjugation of amino acids, which can provide increased bioavailability and reduce the cytotoxicity of the drug *in vivo*. This divergent approach opens up the possibility of generating prodrugs based on gold(I) complexes to target cancer cells, with reduced side effects.

Chemistry

Synthesis of the complexes

We herein report on the synthesis of a series of gold(I) complexes **1–6** (Fig. 2 and 3) that contain amino acids with variable physical properties. The amino acids are ligated to gold(I) by the use of a triflic amide bond and the complexes feature a lipophilic di- or tri-phenylphosphine backbone. Therapeutically

active compounds containing gold amide adducts are not commonplace. Amagai *et al.* have reported nitrogen-containing compounds featuring pyrimidine dione adducts at the surface of a gold nanoparticle, but the nature of the bond is unclear. Importantly, these ligands derive from 5'-fluorouracil, which is therapeutically active as a standalone drug.¹⁴

Two other alternatives have been reported involving the modification of NHC ligands with amino acids and the use of the sulfur-containing cysteine.¹⁵ Our approach, however, allows the use of widely available amino acids and pyridine analogues that are not limited to specific carbene precursors or sulfide complexes. Our complexes offer two advantages over NHC ligands. Firstly, unlike NHCs which are strong σ -donor ligands, the amide linkage offers the possibility for ligand exchange. Secondly, the efficiency and ease with which wide-ranging and varied structures can be generated paves the way for prodrug development and orthogonal therapeutic strategies on multiple targets.

Modification of the X ligand

Complexes **1–3** were synthesized from tyrosine and tryptophan, both aromatic amino acids, and methionine, a non-polar aliphatic amino acid. The precursor ligands featured in complexes **1** and **2** were obtained after esterification of the corresponding amino acids **7** and **8** to their α -amino esters¹¹ followed by triflation of the primary amine with triflic anhydride (Fig. 2).

Complex **3** was synthesised from methionine methyl ester **9** in 3 steps by homologation with succinic anhydride, EDC-mediated amidification with triflic amide and ligand metathesis with the silver amide derived from silver carbonate (Fig. 2).

A different aromatic complex **4** was synthesised from amino-pyridine **10** to contrast the activity of aromatic complexes **1** and **2** with an aromatic zwitterionic complex featuring a delocalised cationic charge. Of note, the pyridyl ligand did not complex gold(I) as its triflic amide, but instead as a pyridinium adduct as established by X-ray crystallography. The pyridinium complex **4** was obtained after mono-triflation of primary amine **10** with triflic anhydride followed by reaction with silver carbonate and gold(I) chloride (Fig. 2).

Modification of the L-ligand

Ligand **11** derived from the polar amino acid proline was used in both complexes **5** and **6** and was synthesized from diphenylphosphine **12** (Fig. 3). Secondary amine **12** was alkylated with methyl 3-bromopropionate¹⁶ and the ester was subsequently saponified to the corresponding carboxylic acid. An EDC-mediated amidification reaction afforded the corresponding triflic propionamide **11**.

Although complexes **5** and **6** feature the same ligand **11** and are derived from the same dimethylsulfide gold chloride complex, the two different structures depend on the initial presence of a silver(I) salt. The unusual dimeric complex **6** was obtained in one synthetic operation by ligand metathesis between a silver amide salt generated *in situ* from ligand **11**

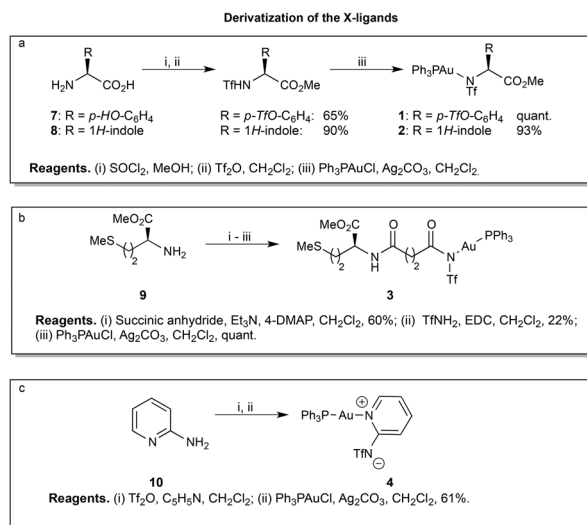


Fig. 2 Modification of the L-ligand of the organogold complexes with biocompatible ligands. The synthesis of Au(I) complexes **1–6** derived from amino acids and amino-pyridine **10**.

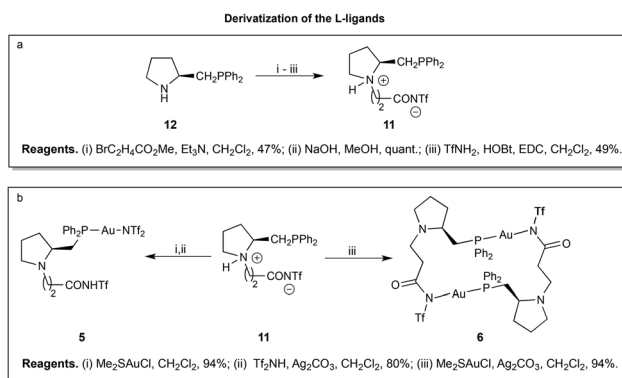


Fig. 3 Modification of the L-ligand of the organogold complexes with biocompatible ligands. The top scheme describes the synthesis of ligand **11** featured in both the mononuclear and the dinuclear organogold complexes **5** and **6**.

and displacement of the more labile dimethylsulfide L-ligand of Me_2SAuCl . Complex 5, in contrast, was generated by L-ligand exchange, isolation of the resulting chloride salt and ligand metathesis with silver bis-triflic amide (Fig. 3).

Results

Cytotoxicity studies

The cell lines CV-1, MDA-MB-231 and MDA-MB-468 were obtained from Cancer Research UK. Cells were grown as described in the ESI.† Cell viability assays, IC_{50} determination, and *in vitro* TrxR and GR assays are described in the ESI.†

In vitro assessment of antiproliferative effects on gold(i) compounds on two breast cancer cell lines

Previously reported gold(i) complexes were tested for antiproliferative effects on two commonly used and well characterised breast cancer cell lines (MDA-MB-231 and MDA-MB-468) as well as CV-1 cells from African green monkey kidney epithelial cells ('control'). CV-1 cells can be grown under the same regime as the two breast cancer cell lines, have been compared to breast (and other) cancer cell lines in numerous studies, and the relationship between mitochondrial activity and DLC accumulation is particularly well-studied in this cell line.^{17,18}

No marked cytotoxicity was observed for Ph_3PAuCl . This was not due to the L-ligand, since Me_2SAuCl was also ineffective in mediating cellular toxicity. The CV-1 cells were unaffected by either precursors at concentrations up to 50 μM . Only at concentrations above 30 μM did we observe a slight effect on the MDA-MB-231; however, at 50 μM , the IC_{50} had not yet been reached for either precursor.

In contrast, $\text{Ph}_3\text{PAuNTf}_2$ displayed preferential inhibition of the two cancer cell lines compared to CV-1 (Fig. 4). This clearly demonstrates that a gold triflic amide linkage can successfully be used in gold complexes to induce cytotoxicity in the cancer cell lines described herein.

Currently, auranofin is undergoing phase II clinical trials for treatment of chronic lymphocytic leukemia (CCL). $\text{Ph}_3\text{PAuNTf}_2$ was found to be as cytotoxic as auranofin (Fig. 4). Although the gold-sulfide has been changed to a gold-selenide,¹⁹ further derivatization of auranofin is limited to either the functionalization of its acetate group or regioselective transesterification to different esters.

Moreover, side effects of auranofin are common and prodrug delivery of the gold(i) is therefore desirable. Using $\text{Ph}_3\text{PAuNTf}_2$ as a model for the amide linkage, we derivatized a number of amino acids, a common mechanism for prodrug formulation.§

Our initial attempts at derivatization of $\text{Ph}_3\text{P-Au-NTf}_2$ involved the two amino acids valine and leucine, which

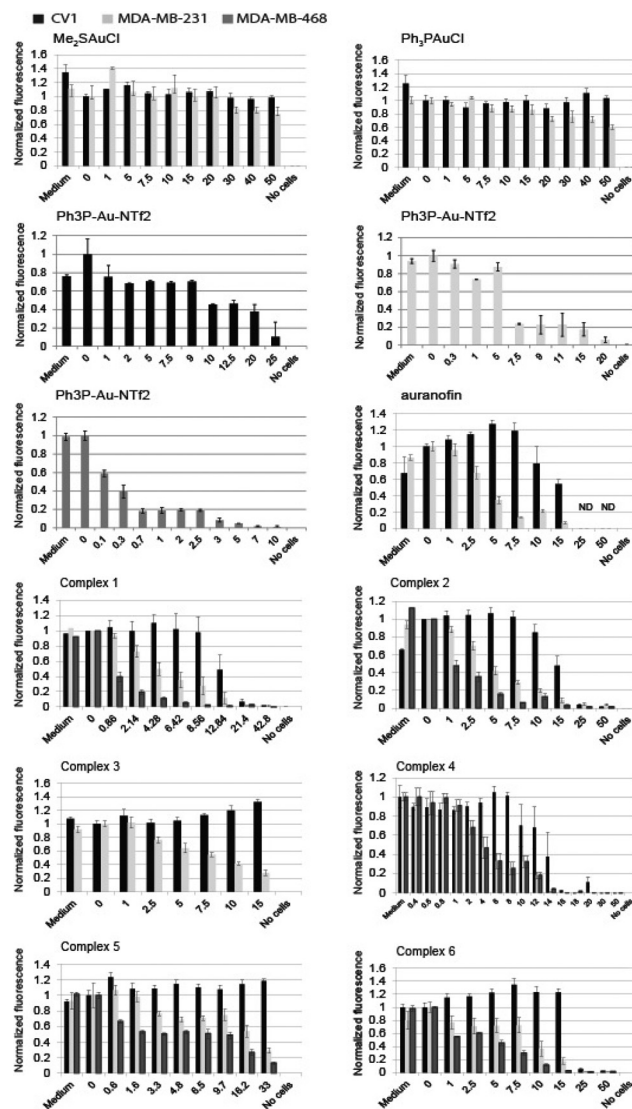


Fig. 4 Cell viability in response to exposure to gold(i) complexes. Cell viability was determined by measuring the fluorescence of untreated ('Medium'), DMSO-treated ('0 μM '), and gold(i) treated cells. The 'No cells' control was used as background subtraction. The normalized fluorescence was calculated as the background-subtracted fluorescence reading, divided by the background-subtracted fluorescence reading of the DMSO-treated control. The error bars correspond to the standard deviation of the mean of four to eight replicates. ND, not done.

contain non-polar aliphatic groups and therefore were predicted to result in highly lipophilic complexes. However, the complexes proved insoluble in an aqueous medium.

Complexes 1 and 2 were derived from aromatic tyrosine and tryptophan, respectively. These complexes contain a heteroatom in the side chain and were soluble. The initial screen of these compounds revealed marked cytotoxic effects on par with $\text{Ph}_3\text{PAuNTf}_2$. The MDA-MB-468 cell line was consistently more sensitive to the gold(i) compounds compared to the MDA-MB-231. As with $\text{Ph}_3\text{PAuNTf}_2$, complexes 1 and 2 were comparable to auranofin in the inhibition of the MDA-MB-231 cell line (Fig. 4).

§For example next-generation valacyclovir was modified by esterification of acyclovir with valine, which improved its bioavailability dramatically.

Further analysis revealed IC_{50} values in the low and even sub-micromolar ranges (ESI Fig. S1 online†). Importantly, the ligands alone did not induce any significant cell toxicity (ESI Fig. S2 online†). Therefore, generating amino acid derivatives of $Ph_3PAuNTf_2$ does not interfere with its cytotoxic effects.

We also investigated the activity of complex 3 derived from methionine to model the use of peptidic bonds as linkages for ligand customisation. This modification also did not impair the cytotoxicity profile (Fig. 4) and clearly shows that derivatizing the NTf_2 X-ligand with amino acid R-groups does not impact on its cytotoxic effects.

The possibility of a delocalised cation in a zwitterionic complex was investigated with complex 4. This contrasts with previous work reported by Hickey *et al.* on delocalised lipophilic cations where two NHC ligands were used in a cationic complex. Here, the use of one X-ligand based on a pyridinium salt concomitantly with a lipophilic triphenylphosphine successfully mediated cellular toxicity in the two cancer cell lines, without affecting CV-1 cells. The ligand alone produced no cytotoxic effect (ESI Fig. S2 online†). These data show that the gold-amide ligand effects are substantial and that the derived ligands significantly influence the efficacy of the gold(i) complexes in mediating cellular toxicity.

To rationalise the effect of the phosphine ligand, pyrrolidine **11** was used as an L-ligand by formally replacing a phenyl ring with a modified proline, as seen in complex 5. Interestingly, this change in the stereoelectronic and physical properties of the L-ligand did not impact on its cytotoxicity against either of the cancer cell lines when compared to $Ph_3P-Au-NTf_2$. This broadens the possibilities for further modification of the gold complexes (Fig. 4).

We have here established that, as long as a triflic amide is used as an X-ligand, amino acids can also be derivatised to serve as L-ligands in gold amide complexes without affecting the cytotoxicity of the complex. It should therefore be possible to modulate the polarity of L-ligands, which can in turn be used as prodrugs or modulators for cytotoxicity.

Finally, we tested the potential of a dinuclear bis-gold(i) complex **6** in its efficacy to kill cancer cells, but the results were similar to that of complex 3 and increased activity was not observed.

Preferential sensitization of CV-1 cells to gold(i) complexes with treatment of nigericin

Mitochondrial hyperpolarization of adenocarcinomas from the two cell lines used in this study is hypothesized to be the cause of the selective inhibition of cell proliferation due to enhanced accumulation of gold(i) in the mitochondria.^{5,17} This predicts that hyperpolarizing the mitochondrial membranes of CV-1 cells with the K^+/H^+ ionophore, nigericin, should sensitize them further to the gold complexes.^{20,21} To test this hypothesis, CV-1 cells were treated with increasing concentrations of nigericin in addition to 0 or 4.28 μM of complex 1 or 0 or 5 μM of complex 2. Nigericin treatment alone caused a concentration-dependent decrease in cell viability.

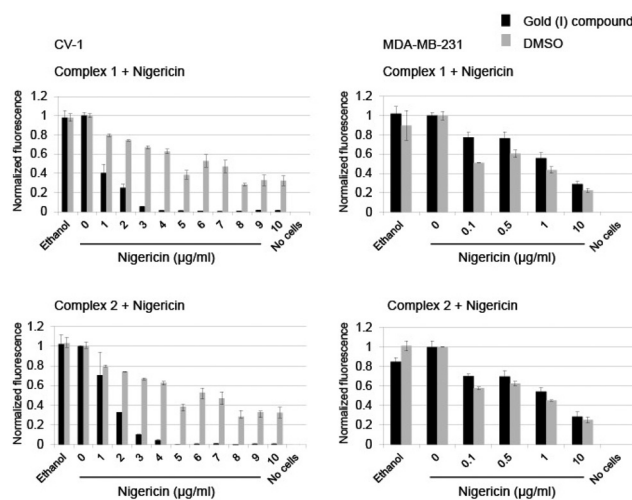


Fig. 5 Nigericin sensitizes CV-1 cells to gold(i) compounds. CV-1 and MDA-MB-231 cell lines were treated with increasing concentrations of nigericin in addition to 4.28 or 5 μM gold(i) complex (black bars) or 0 μM gold(i), DMSO-treatment (grey bars). Ethanol-only treatment of the cell lines (no gold(i) compounds) was used as 'Ethanol' control. 'No cells' control was used for background subtraction. The error bars represent standard deviation of the mean of three replicates.

When used in combination with complexes **1** or **2**, nigericin caused a marked further sensitization to the aforementioned complexes (Fig. 4). In contrast, although MDA-MB-231 cells also showed a degree of sensitivity to nigericin alone, the addition of complexes **1** or **2** caused no further decrease in cell viability (Fig. 5).

Complexes **1** and **2** inhibit TrxR but not GR *in vitro*

A selenocysteine-containing protein, thioredoxin reductase (TrxR), has been demonstrated to be inhibited by gold(i) complexes *via* coordination of the transition metal to the selenocysteine residue.^{5,22,23} Increasing concentrations of complexes **1** and **2** inhibited the TrxR enzyme in the nM range *in vitro* (Fig. 6). Treatment with solely the ligands did not inhibit TrxR, demonstrating that gold(i) is required for the inhibition of TrxR. The related protein, glutathione reductase (GR), which contains a cysteine, was unaffected, suggesting that the gold(i) complexes selectively inhibit proteins containing a selenol function.^{5,12,24}

Conclusions

In our search for therapeutically active gold(i) complexes featuring an amide linkage stable enough to not dissociate in solution *in vivo*, yet retaining the therapeutic activity of gold(i), we envisaged utilising the complexes described in Fig. 1. These contain a gold amide linked to an electron withdrawing group and a side group. We have herein reported on their synthesis and derivatisation with robust, simple and high-yielding routes which are amenable to high-throughput synthesis. The ligands themselves did not impair the activity of the

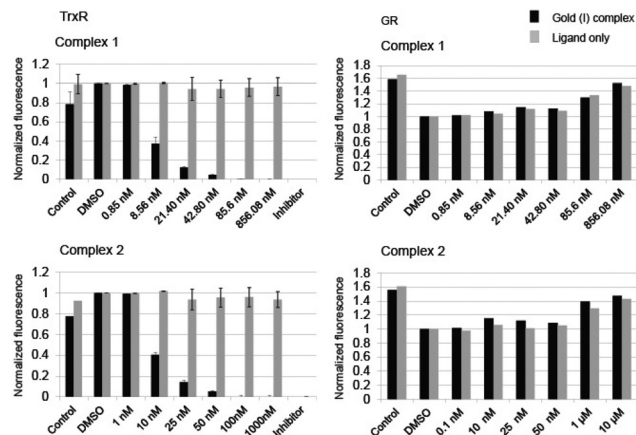


Fig. 6 TrxR activity *in vitro* is inhibited by gold(I) complexes. Increasing concentrations of complexes **1** and **2** or 'ligand only' were added to TrxR and GR enzymes and their activity was monitored by fluorescence. Normalized fluorescence values were calculated by taking the fluorescence value of the treated well and dividing it by the fluorescence value of 0 nM compound (DMSO-only treatment).

complexes, and were shown to have no cytotoxic effects on their own. We have also demonstrated the compatibility of a wide array of functionalities such as indoles, phenol triflates, esters, methyl sulfide, amides, aliphatic chains, aromatic groups, diarylalkyl- and triaryl-phosphines. An alternative chemical environment that was equally active as the gold triflic amide complexes was also validated. The use of a zwitterionic ligand bearing a delocalised cation clearly sets a precedent for the use of therapeutically active analogues of globally electro-neutral pyridinium gold(I) complexes in treating cancer. The two approaches described herein open up new possibilities for an increased diversity in new chemotypes and prodrug delivery, including peptoid and peptidic precursors as well as orthogonal anticancer drugs.

The selective accumulation of the gold(I) complexes in adenocarcinoma cells due to their mitochondrial hyperpolarization may provide a favourable therapeutic index for the treatment of tumours. This opens up the possibility of targeting cancers in elderly patients and of slow-growing tumours, where the conventional cancer therapeutics aimed at rapidly dividing cells cannot be used.

Funding sources

E.H. is the recipient of an MRC Senior Fellowship and EMBO Young Investigator Award. E.M.E.V. is funded by an Alfred Bader award.

Acknowledgements

We thank Dr Iain Day for the NMR service and Dr Alaa Abdul-Sada for the Mass Spectrometry Service at the University of Sussex; the NCS at Southampton for X-ray data for compound

4; and Dr Peter Karran (CRUK) for cell lines and Dr Judith Offman for setting up cell lines. We also thank Prof. M. Bagley, Prof. P. J. Parsons, Prof. S. Ward and Drs O. Navarro and J. Spencer for their continued interest in this work. We also wish to thank Chris Dadswell and Aidan Fisher for their work on the ICP-MS studies and lipophilicity determination respectively.

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